

Cloning of a novel putative G-protein-coupled receptor (NLR) which is expressed in neuronal and lymphatic tissue

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Received 15 March 1993

A novel G-protein-coupled receptor was isolated from mouse and rat neuronal and lymphatic tissues. The amino acid sequence of the rat receptor (rNLR) shows an overall homology of 80% to a recently cloned receptor from Burkitt's lymphoma cells (BLR1) which is exclusively expressed in lymphatic tissues [(1992) *Eur. J. Immunol.* 22, 2795]. Much less homology between rNLR and BLR1 was observed at the N-terminus (about 40%), whereas rNLR and the mouse homologue mNLR show 92% amino acid identity. Northern blot analysis of NLR revealed a predominant 5.5 kb mRNA species in various brain regions and neuronal cell lines, whereas in the spleen a 3 kb transcript is predominant. This distribution suggests a role of NLR in the nervous and immune systems.

G-protein-coupled receptor; NLR; NG108-15 cell; Polymerase chain reaction; BLR1; Burkitt's lymphoma; Interleukin 8

1. INTRODUCTION

G-protein-coupled receptors are activated by hormones, neurotransmitters or neuropeptides and transduce these signals into the cell by specific interaction with GTP binding proteins. Recent evidence suggests that some members of the cytokine family also produce their effects by interacting with G-protein-coupled receptors [1]. In particular, interleukin-8 (IL-8), which belongs to a family of structurally related cytokines similar to platelet factor 4, exerts its effect through a G-protein-coupled receptor [2,3]. Sequence homology between the cloned G-protein-coupled receptors indicates a high degree of similarity of the high- and low-affinity IL-8 receptors with the receptors for chemoattractants, such as fMet-Leu-Phe, C5a and, surprisingly, also for neuropeptide receptors, such as the delta opiate receptor (DOR-1) [4], neuropeptide Y-3 receptor [5] and the somatostatin-2 receptor [6]. Furthermore, a novel member of a G-protein-coupled receptor (BLR1) has been cloned from Burkitt's lymphoma cells with 37% homology to the low affinity IL-8 receptor and shown to be exclusively expressed in lymphatic tissue [7].

In the present study we describe the cloning of NLR, a putative G-protein-coupled receptor which exhibits a high homology to BLR1 and, in contrast to BLR1, is expressed not only in lymphatic tissues such as the

spleen but also in various brain areas and in neuronal cell lines.

2. MATERIALS AND METHODS

2.1. Poly(A) RNA preparation and Northern blot analysis

Total RNA was isolated by the acid guanidinium isothiocyanate method [8] and poly(A) RNA was selected by QuickPrep columns (Pharmacia, Freiburg, Germany). After electrophoresis the RNA was blotted onto Nytran membranes (Schleicher & Schüll, Germany) and hybridized with a [³²P]UTP-labeled cRNA probe according to standard methods [9].

2.2. Polymerase chain reaction, cloning and sequencing

Polymerase chain reaction (PCR) to amplify cDNA's encoding potential G-protein-coupled receptors was performed as follows: first strand cDNA for PCR reaction was generated from 1 µg NG108-15 cell poly(A) RNA using an oligo(dT) primer and MoMLV reverse transcriptase (SuperScript, BRL) in 20 µl final reaction volume. 1 µl of reaction was amplified by 30 cycles of PCR [10] using primers derived from transmembrane segments III and VI [11]. Cycle conditions were 1.5 min at 95°C, 2 min at 50°C and 2 min at 72°C. The resulting products were purified with PrimeErase columns (Stratagene), blunted with Klenow polymerase and cloned into pBluescript (Stratagene). Dideoxynucleotide sequencing was then performed by the chain termination method [12] with Sequenase-2.0 (USB). Four clones showing the motif of G-protein-coupled receptors were obtained (561-45, 561-51, 561-39, 561-8).

2.3. Library screening and subcloning

Clone 561-8 was ³²P-labelled by the random primer technique [13] and used as a probe to screen a rat genomic library constructed in phage λEMBL3 and a NG108-15 hexanucleotide-primed cDNA library in λZAP II (custom synthesis by Invitrogen, Heidelberg, Germany). Out of 5 × 10⁵ genomic phage recombinants screened, three identical clones were isolated and subjected to restriction mapping and Southern blot analysis. A 7 kb *Bam*HI fragment containing the full coding region was subcloned in pBluescript. Out of 1 × 10⁶ recombinant NG108-15 cDNA phages screened only one 3' truncated clone, designated N18-10, was isolated.

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In order to isolate the rat N-terminal Exon (EX1), primer extension and second strand synthesis was performed on 1 μ g rat spleen mRNA with primer SPR-3 (Fig. 1B) according to the protocol of reverse transcriptase Superscript (BRL). The resulting products were cloned in pBluescript and verified by colony hybridization and sequencing.

2.4. *In situ* hybridization

A sagittal section of rat brain was hybridized with a [35 S]UTP-labelled antisense RC8 RNA probe and for control with a sense RNA probe, under conditions previously described [14].

2.5. Stable expression and ligand binding

The cDNA clone RC8 was subcloned in pRC/CMV (Invitrogen, Heidelberg, Germany) and transfected into CHO-K1 cells by Lipofectin (BRL). Individual clones were isolated by limiting dilution in DMEM-F12-medium (BRL) with 600 μ g/ml G418. Two clones expressing the highest levels of RC8 mRNA were used for binding experiments as described [17].

3. RESULTS AND DISCUSSION

Poly(A) RNA from NG108-15 cells, a hybrid cell line of mouse N18TG2 neuroblastoma and rat C6BU-1 glioma parent cell lines, was subjected to PCR amplification with degenerate primers to transmembrane domain III and VI of G-protein-coupled receptors as described [11]. Sequence determination of cloned PCR fragments revealed several putative receptor-encoding cDNA clones. Clone 561-45 was identical to rat endothelin 1A receptor [15] and clone 561-51 shows sequence identity to the mouse somatostatin 2 receptor [16]. Screening of a NG108-15 library with this partial cDNA resulted in the cloning of an isoform of the SST2 receptor (SSTR2B) [17]. In addition several novel putative receptor fragments were identified (data not shown). One cDNA fragment (561-8) was used as a hybridization probe to screen a rat genomic library. Restriction analysis of several positive phage clones led to the isolation of a 7 kb *Bam*HI fragment named λ RG8 (Fig. 1A). This fragment contains the coding region from membrane domain I-VII plus the stop codon, but lacks a hydrophilic N-terminal extracellular domain and a start codon, indicating the existence of an intervening sequence. Therefore a cDNA library was constructed from rat spleen mRNA by primer extension using a

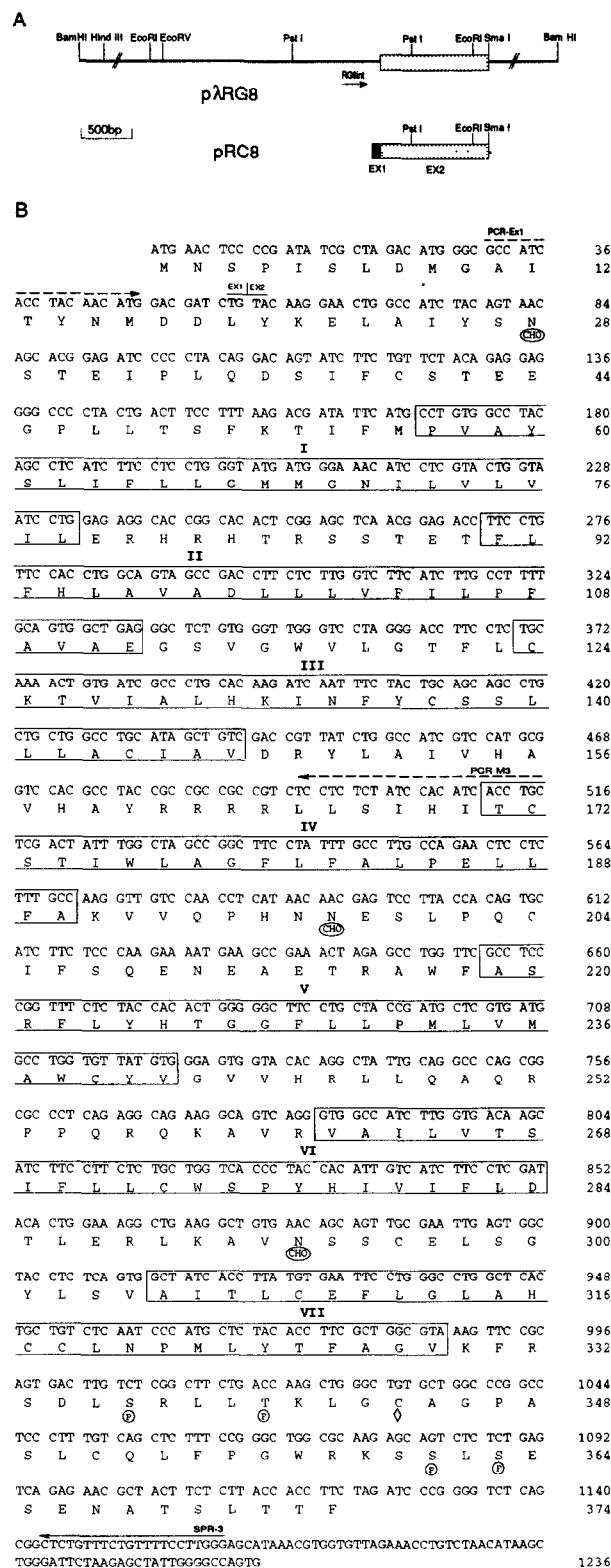


Fig. 1. (A) Organization and restriction map of λ RG8 rat genomic clone and RC8 rat spleen cDNA clone. The thick line represents genomic sequences, the shaded box the exon 2, and the black box the exon 1 only present in RC8. RG8int represents an intron-specific primer 122 bp upstream of the exon 2 acceptor site used for PCR. (B) Nucleotide and amino acid sequence of rNLR as deduced from RC8 and λ RG8 sequences downstream of the SPR-3 primer. The putative membrane-spanning domains are boxed and are assigned on the basis of Kyte and Doolittle hydrophobicity plot [25]. Potential N-linked glycosylation sites (CHO), potential phosphorylation recognition sites (P) [26] and the potential palmitoylation site at the C-terminus (◇) are indicated. Primers used for reverse transcription (SPR-3) and PCR (PCR-Ex1, PCR-M3) are marked by a solid line and dashed lines with arrows, respectively. The intron-exon junction is marked.



λ RG8-specific primer (SPR-3; Fig. 1B). Sequencing of λ RG8-positive colonies revealed a cDNA clone RC8, which is identical to the transmembrane domains I–VII of IRG8 and has additional sequences at the N-terminus, including several methionines as putative start codons (Fig. 1A).

The longest open reading frame of RC8 cDNA specifies a sequence of 1,122 bp corresponding to a protein of 374 amino acids with seven highly hydrophobic regions (Fig. 1B), designated rNLR (rat neurolymphatic receptor). The protein sequence of rNLR predicts potential N-linked glycosylation sites in the N-terminal extracellular region as well as in the second and third extracellular loop. In addition, several serine and threonine residues are located between transmembrane domains III and IV and within the C-terminal region and may represent potential phosphorylation sites. A conserved cysteine residue (Fig. 1B, residue number 344) may function as an acceptor site for palmitoylation [18].

An homology search on protein databases reveals the

highest similarity of rNLR to BLR1 (80%), a G-protein-coupled receptor recently isolated from Burkitt's lymphoma cells [7] (Fig. 2). In addition, rNLR shows significant homologies to the low and high affinity receptors for IL8 (IL-8Rb, IL-8Ra, 37%, 35%) [19], a bovine neuropeptide Y receptor (NPY3, 35%) [5], the vascular type-1 angiotensin II receptor (rVAT II R, 30%) [20] and the rat somatostatin 2 receptor (rSSTR2, 26%) [6]. rNLR also exhibits similarities to the receptors for fMet-Leu-Phe (FMLR, 25%) [21] and human C5a anaphylatoxin (C5AR, 24%) [22]. All these receptors contain common features, such as the conserved proline residues in transmembrane regions IV, V, VI, and VII, known to function as helix initiators and thought to surround the ligand binding pocket within the membrane; furthermore cysteine residues are present in the second and third extracellular loops, which may be involved in the formation of disulfide bridges [18].

In addition, we have isolated a 3'-truncated cDNA clone from a NG108-15 library, termed N18-10, encod-

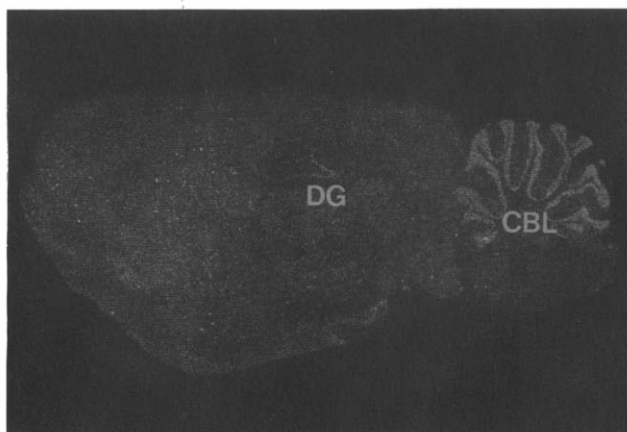


Fig. 5. Localization of NLR receptor mRNA by in situ hybridization. A sagittal section of rat brain was hybridized with an antisense RC8 cRNA probe, labelled with [35 S]UTP. The slices were exposed for 4 days at -70°C with an intensifying screen. Areas with specific mRNA expression are indicated. CBL, cerebellum; DG, dentate gyrus. Hybridization with the sense probe shows no specific signals (data not shown).

cortex rNLR is also present in the striatum, the pituitary and adrenal gland. Since rat C6 glioma cells do not express the NLR receptor, the mRNA present in NG108-15 mouse neuroblastoma \times rat glioma hybrid cells appears to derive exclusively from the mouse neuroblastoma parent cell line, N18TG2.

The existence of NLR mRNA in the brain is also demonstrated by in situ hybridization experiments (Fig. 5). In the sagittal section of a rat brain, hybridization signals with an antisense probe were observed predominantly over cell layers of the cerebellum. In addition, a lower expression level is found over the granule cells of the dentate gyrus of the hippocampus. This localization of NLR mRNA is in good accordance with the observation that neuronal cells, but not glia cells, express the receptor in NG108-15 cells.

Taken together, rNLR and its mouse analogue represent putative G-protein-coupled receptors which are expressed in neuronal and lymphatic tissue. They share a high homology to a recently cloned receptor from Burkitt's lymphoma cells (BLR1) [7] and hybridize to the same 3 kb mRNA species in spleen. In other tissues, however, a 5.5 kb NLR transcript is predominant. The nature of the 5.5 kb mRNA is presently not known. The NLR gene, like the BLR1 gene, contains an intervening sequence between the N-terminus and the first transmembrane domain. In the BLR1 gene this intron comprises 9 kb, and a similar intron size has been found for the mouse analogue of the gene (M. Lipp, personal communication). The possibility of alternatively spliced mRNA species within this intron can be excluded, since a single amplification product was obtained by PCR with intron-flanking primers and cDNAs from rat tissues and NG108-15 cells. In addition, the N-terminus of rNLR which has been cloned from rat spleen, is

almost identical to the N-terminus of its mouse analogue, which has been cloned from mouse neuroblastoma cells (N18TG2), indicating that spleen and neuronal cells may express a NLR mRNA with similar 5' coding regions. We argue that the 5.5 kb mRNA may represent a 5'-extended species, which derives from different transcription initiation sites of the gene.

Although rNLR and its mouse analogue share a high overall sequence homology to the human BLR1, the homology at the N-terminus is much less pronounced (Fig. 3). A similar situation is observed in the case of the high- and low-affinity IL-8 receptors which exhibit a high homology except in the N-terminal region. Therefore, we suggest that this N-terminal divergence between rat/mouse NLR vs. human BLR1 may not reflect species differences. Instead NLR and BLR1 may represent homologous receptors that differ in their affinity for a common ligand or have structurally related ligands. This hypothesis is also supported by the observation that BLR1 has 11 negative amino acid residues in the extracellular N-terminus, whereas both rNLR and mNLR have 7 negatively charged amino acids (Fig. 3), thought to play an important role for the IL-8 receptor in binding of the basic ligand [3].

In order to determine the ligand for NLR we stably expressed the clone, RC8, under the control of the CMV promotor in CHO-K1 cells. Two independent clonal cell lines expressing the highest level of RC8 mRNA (data not shown) were tested for binding with the following ligands: [^3H]CCK-8 (cholecystokinin-8), [^3H]DADLE (D-Ala-D-Leu-enkephalin), [^3H]angiotensin II, [^3H]oxytocin and [^{125}I]11-Tyr-somatostatin. None of these ligands showed binding to either NLR-expressing clonal CHO lines.

The homology of the putative G-protein-coupled receptor NLR to both the cytokine and neuropeptide family of receptors raises the question of its biological importance in neuroimmune connections. Several neuropeptides, such as neuropeptide Y, calcitonin gene-related peptide, galanin and opioids, besides their wide distribution throughout the central and peripheral nervous system, have been demonstrated to be localized in nerve endings innervating lymphoid organs [23]. Interestingly, neuropeptide Y-positive nerve terminals were found in close apposition with lymphoid compartments of the rat spleen [24]. Thus, the precise localization of NLR expression in the lymphoid compartments of the spleen, as well as the identification of its ligand, may contribute to our understanding of neuroimmunomodulation.

Acknowledgements: The authors thank Dr. B. Hamprecht (Tübingen, Germany) for the NG108-15 cell line, Dr. Scherer (Freiburg, Germany) for the rat genomic EMBL3 library, Dr. J. Johnson (Munich, Germany) for providing us with RNA from mouse spleen and thymus and for the Raji cell line, and Dr. M. Lipp (Martinsried, Germany) for helpful discussions. This work was supported by DFG SPP 'Neuropeptide'.

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